08-29-01 533 Rec'd PCT/PTO 28 AUG 2001 PCT $

FORM PTO-1390 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

08/28/01

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
PA-9902	09/914603

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB00/00807	March 9, 2000	March 12, 1999

TITLE OF INVENTION

Analysis of Differential Gene Expression

APPLICANT(S) FOR DO/EO/US

Nicholas Thomas and Alan Waggoner

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

    copy of this transmittal letter for charging purposes
    return postcard

APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/914603	PCT/GB00/00807	PA-9902

24. ... following fees are submitted:.

		CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :			
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1000.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$860.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$690.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	17 - 20 =	0	x $18.00	**$0.00**	
Independent claims	1 - 3 =	0	x $80.00	**$0.00**	
Multiple Dependent Claims **(check if applicable).**			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$860.00**	
☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$860.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$860.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			☐	**$0.00**	
TOTAL FEES ENCLOSED =				**$860.00**	
				Amount to be: refunded	$
				charged	$

a. ☐ A check in the amount of ______________ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **500-588** in the amount of **$860.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **500-588** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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REGISTRATION NUMBER

August 28, 2001

DATE

09/914603
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PA-9902

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	N. Thomas, et al.	Group Art Unit:	To be assigned
Serial Number:	To be assigned	Examiner:	To be assigned
Filing Date:	August 28, 2001		
Title:	Analysis of Differential Gene Expression		

FIRST PRELIMINARY AMENDMENT

Honorable Assistant Commissioner of Patents
Box Patent Application
Washington, D.C. 20231

Sir:

Please consider the following amendments and remarks in connection with the prosecution of the captioned application, which is a filing under 35 U.S.C. § 371 and claims priority to international application number PCT/GB00/00807 filed March 9, 2000. This application also claims priority to application number 9905807.5 filed in Great Britain on March 12, 1999.

In the Claims

Please amend page 13, line 1 as follows:

[CLAIMS]

What is claimed is:

Please amend claim 1 as follows:

1. (once amended) A method of detecting and analysing differences between nucleic acids from two sources, which method comprises:

   a. providing the nucleic acids from two sources as labelled probes wherein the nucleic acids from two sources are labelled with two different markers;

   b. forming a mixture of the labelled probes with pooled reagents wherein each of the pooled reagents comprises[reagent is] a population of beads carrying a polynucleotide target, the polynucleotide target of any one of the pooled reagents[reagent] being different from the target of [another reagent,]any other of the pooled reagents and the beads of any one of the pooled reagents[reagent] being distinguishable from the beads of [another reagent]any other of the pooled reagents;

   c. incubating the mixture under conditions to promote specific hybridisation between probes and targets; and

   d. analysing beads in the mixture by flow cytometry.

Please amend claim 3 as follows:

3. (once amended) The method of claim 1[or claim 2] wherein the polynucleotide targets are cDNA derived from cellular mRNA.

Please amend claim 4 as follows:

4. (once amended) The method of [any one of claims 1 to 3]claim 1 wherein the polynucleotide targets are PCR amplimers.

Please amend claim 5 as follows:

5. (once amended) The method of [any one of claims 1 to 4]claim 1 wherein the polynucleotide targets [carry]contain terminal biotin groups through which they are attached to streptavidin-coated beads.

Please amend claim 6 as follows:

6. (once amended) The method of [any one of claims 1 to 5]claim 1 wherein the polynucleotide targets are single-stranded nucleic acids.

Please amend claim 7 as follows:

7. (once amended) The method of [any one of claims 1 to 6]claim 1 wherein the [labelled probes]nucleic acids are single-stranded nucleic acids.

Please amend claim 8 as follows:

8. (once amended) The method of [any one of claims 1 to 7]claim 1 wherein beads of one pooled reagent are distinguishable from beads of another pooled reagent by size.

Please amend claim 9 as follows:

9. (once amended) The method of [any one of claims 1 to 8]claim 1 wherein beads of one pooled reagent are distinguishable from beads of another pooled reagent by the nature of [the]one or more markers attached to the beads.

Please amend claim 10 as follows:

10. (once amended) The method of [any one of claims 1 to 9]claim 1 wherein beads of one pooled reagent are distinguishable from beads of another pooled reagent by the concentration of one or more markers attached to the beads.

Please amend claim 11 as follows:

11. (once amended) The method of [any one of claims 1 to 7]claim 1 wherein beads

of one pooled reagent are distinguishable from beads of another pooled reagent by the size and/or by the nature [and/or]and the concentration of one or more markers attached to the beads.

Please amend claim 12 as follows:

12. (once amended) The method of [any one of claims 8 to 11]claim 9 wherein the markers are fluorescent markers [are]attached to the beads.

Please amend claim 13 as follows:

13. (once amended) The method of claim1[or claim 2] wherein each [probe]of the nucleic acids is labelled with a fluorescent tag to indicate its source.

Please amend claim 14 as follows:

14. (once amended) The method of [any one of claims 1 to 13]claim 1 wherein the analysis by flow cytometry is performed to identify each bead and to quantify the probes bound thereto.

Please amend claim 15 as follows:

15. (once amended) The method of [any one of claims 1 to 14]claim 1 [wherein]further comprising the step of analysing the data obtained by flow cytometry [is analysed]to yield information about the relative and/or absolute abundances of individual nucleic acid sequences [of]contained within the nucleic acids from [the]two sources.

Please add new claim 16 as follows:

16. (new) The method of claim 10 wherein the markers are fluorescent markers attached to the beads.

Please add new claim 17 as follows:

17. (new) The method of claim 11 wherein the markers are fluorescent markers attached to the beads.

Remarks

Claims 1-15 are pending in the instant application. Applicants have amended

claims 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 to more fully conform with U.S. practice and to delete multiple dependencies. Applicants have also added new claims 16 and 17. A version of the claims marked up to show the amendments, as well as a clean version of the claims encompassing the amendments, is attached hereto.

Applicants respectfully assert that all amendments are fairly based on the specification, and respectfully request their entry.

Applicants believe that the claims, as amended, are in allowable form, and earnestly solicit the allowance of claims 1-17.

Respectfully submitted,

Royal N. Ronning, Jr. 32,529
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Claims (marked-up version showing amendment(s))

Page 13, line 1:

[CLAIMS]

What is claimed is:

1. (once amended) A method of detecting and analysing differences between nucleic acids from two sources, which method comprises:

   a. providing the nucleic acids from two sources as labelled probes wherein the nucleic acids from two sources are labelled with two different markers;

   b. forming a mixture of the labelled probes with pooled reagents wherein each of the pooled reagents comprises[reagent is] a population of beads carrying a polynucleotide target, the polynucleotide target of any one of the pooled reagents[reagent] being different from the target of [another reagent,]any other of the pooled reagents and the beads of any one of the pooled reagents[reagent] being distinguishable from the beads of [another reagent]any other of the pooled reagents;

   c. incubating the mixture under conditions to promote specific hybridisation between probes and targets; and

   d. analysing beads in the mixture by flow cytometry.

3. (once amended) The method of claim 1[or claim 2] wherein the polynucleotide targets are cDNA derived from cellular mRNA.

4. (once amended) The method of [any one of claims 1 to 3]<u>claim 1</u> wherein the polynucleotide targets are PCR amplimers.

5. (once amended) The method of [any one of claims 1 to 4]<u>claim 1</u> wherein the polynucleotide targets [carry]<u>contain</u> terminal biotin groups through which they are attached to streptavidin-coated beads.

6. (once amended) The method of [any one of claims 1 to 5]<u>claim 1</u> wherein the polynucleotide targets are single-stranded nucleic acids.

7. (once amended) The method of [any one of claims 1 to 6]<u>claim 1</u> wherein the [labelled probes]<u>nucleic acids</u> are single-stranded nucleic acids.

8. (once amended) The method of [any one of claims 1 to 7]<u>claim 1</u> wherein beads of one <u>pooled</u> reagent are distinguishable from beads of another <u>pooled</u> reagent by size.

9. (once amended) The method of [any one of claims 1 to 8]<u>claim 1</u> wherein beads

of one pooled reagent are distinguishable from beads of another pooled reagent by the nature of [the]one or more markers attached to the beads.

10. (once amended) The method of [any one of claims 1 to 9]claim 1 wherein beads of one pooled reagent are distinguishable from beads of another pooled reagent by the concentration of one or more markers attached to the beads.

11. (once amended) The method of [any one of claims 1 to 7]claim 1 wherein beads of one pooled reagent are distinguishable from beads of another pooled reagent by the size and/or by the nature [and/or]and the concentration of one or more markers attached to the beads.

12. (once amended) The method of [any one of claims 8 to 11]claim 9 wherein the markers are fluorescent markers [are]attached to the beads.

13. (once amended) The method of claim1[or claim 2] wherein each [probe]of the nucleic acids is labelled with a fluorescent tag to indicate its source.

14. (once amended) The method of [any one of claims 1 to 13]claim 1 wherein the analysis by flow cytometry is performed to identify each bead and to quantify the probes bound thereto.

15. (once amended) The method of [any one of claims 1 to 14]claim 1 [wherein]further comprising the step of analysing the data obtained by flow cytometry [is analysed]to yield information about the relative and/or absolute abundances of individual nucleic acid sequences [of]contained within the nucleic acids from [the]two sources.

16. (new) The method of claim 10 wherein the markers are fluorescent markers attached to the beads.

17. (new) The method of claim 11 wherein the markers are fluorescent markers attached to the beads.

Claims (clean version encompassing amendments)

What is claimed is:

1. (once amended) A method of detecting and analysing differences between nucleic acids from two sources, which method comprises:

   a. providing the nucleic acids from two sources as labelled probes wherein the nucleic acids from two sources are labelled with two different markers;

   b. forming a mixture of the labelled probes with pooled reagents wherein each of the pooled reagents comprises a population of beads carrying a polynucleotide target, the polynucleotide target of any one of the pooled reagents being different from the target of any other of the pooled reagents and the beads of any one of the pooled reagents being distinguishable from the beads of any other of the pooled reagents;

   c. incubating the mixture under conditions to promote specific hybridisation between probes and targets; and

   d. analysing beads in the mixture by flow cytometry.

2. The method of claim 1 wherein the nucleic acids from two sources are mRNA or cDNA from cells or tissues.

3. (once amended) The method of claim 1 wherein the polynucleotide targets are cDNA derived from cellular mRNA.

4. (once amended) The method of claim 1 wherein the polynucleotide targets are PCR amplimers.

5. (once amended) The method of claim 1 wherein the polynucleotide targets contain terminal biotin groups through which they are attached to streptavidin-coated beads.

6. (once amended) The method of claim 1 wherein the polynucleotide targets are single-stranded nucleic acids.

7. (once amended) The method of claim 1 wherein the nucleic acids are single-stranded nucleic acids.

8. (once amended) The method of claim 1 wherein beads of one pooled reagent are distinguishable from beads of another pooled reagent by size.

9. (once amended) The method of claim 1 wherein beads of one pooled reagent are distinguishable from beads of another pooled reagent by the nature of one or more

markers attached to the beads.

10. (once amended) The method of claim 1 wherein beads of one pooled reagent are distinguishable from beads of another pooled reagent by the concentration of one or more markers attached to the beads.

11. (once amended) The method of claim 1 wherein beads of one pooled reagent are distinguishable from beads of another pooled reagent by the size and/or by the nature and the concentration of one or more markers attached to the beads.

12. (once amended) The method of claim 9 wherein the markers are fluorescent markers attached to the beads.

13. (once amended) The method of claim1 wherein each of the nucleic acids is labelled with a fluorescent tag to indicate its source.

14. (once amended) The method of claim 1 wherein the analysis by flow cytometry is performed to identify each bead and to quantify the probes bound thereto.

15. (once amended) The method of claim 1 further comprising the step of analysing the data obtained by flow cytometry to yield information about the relative and/or

absolute abundances of individual nucleic acid sequences contained within the nucleic acids from two sources.

16. (new) The method of claim 10 wherein the markers are fluorescent markers attached to the beads.

17. (new) The method of claim 11 wherein the markers are fluorescent markers attached to the beads.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68	A2	(11) International Publication Number: WO 00/55363 (43) International Publication Date: 21 September 2000 (21.09.00)

(21) International Application Number: PCT/GB00/00807

(22) International Filing Date: 9 March 2000 (09.03.00)

(30) Priority Data:
9905807.5 12 March 1999 (12.03.99) GB

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

J01

(54) Title: ANALYSIS OF DIFFERENTIAL GENE EXPRESSION

(57) Abstract

The invention provides methods for detecting the differential expression or presence of two analytes, and more specifically to procedures which provide for rapid and efficient analysis of gene expression in biological systems. In particular, the invention provides a method of detecting and analysing differences between nucleic acids from two sources, which method comprises: a. providing nucleic acids from two sources as labelled probes; b. forming a mixture of the labelled probes with pooled reagents wherein each reagent is a population of beads carrying a polynucleotide target, the target of one reagent being different from the target of another reagent, the beads of one reagent being distinguishable from the beads of another reagent; c. incubating the mixture under conditions to promote specific hybridisation between probes and targets; and, d. analysing beads in the mixture by flow cytometry.

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ANALYSIS OF DIFFERENTIAL GENE EXPRESSION

This invention relates to methods for detecting the differential expression or presence of two analytes, and more specifically to procedures which provide for rapid and efficient analysis of gene expression in biological systems.

Analysis of cellular gene expression is key to gaining understanding of the function of biological organisms and to elucidating the mechanisms which control key cellular events, knowledge of which is crucial to the development of drugs and strategies for treatment of disease states arising from disruption of cellular control processes.

A wide variety of methods have been developed for analysing gene expression, both at the level of mRNA expression, or by examining the amounts of specific proteins present in cells. Most methods have the same experimental basis in that they examine differential expression; that is they examine the difference in expression of one or more cellular components between two cells which have been exposed to different conditions. Such studies typically compare expression levels in cells of an untreated population (the control cells) with expression in a separate population of the same cell type (the test cells) that have been subjected to some form of stimulus, for example exposure to a hormone, drug or other chemical.

Early methods for the analysis of differential gene expression were predominantly based on analysis of (often anonymous) mRNA bands on electrophoresis gels. Such approaches have largely been displaced by more powerful, reproducible and informative methods based on the use of arrays of nucleic acids in which large numbers of specific sequences are laid down in an ordered pattern on a solid surface and form the target for hybridisation and capture of labelled mRNA or cDNA from the cells under study. Such arrays have been constructed on a variety of supports, ranging from nylon membranes to glass and silicon wafers. Whatever the support, the essential method of use is the same: firstly known sequences, complementary to cellular mRNAs, either in the form of synthetic oligonucleotides or as PCR products, are laid down on the solid support in spots at defined locations. These immobilised sequences (targets) are then exposed to sequences (probes) extracted from the cells or tissue under study, where the probes are tagged with some form of label which can be detected in subsequent analysis.

In early techniques, radioactive labels were used to probe targets on nylon membranes using techniques developed from Southern and Northern blotting methods. However these methods required the hybridisation process to be performed twice, once for the control sample and once for the test sample. They were subsequently overtaken by more elegant procedures based on the use of different coloured fluorophors to label control and test probes and using either PCR products coupled to glass (Schena M. *et al*, (1996), Proc. Natl. Acad. Sci., 93 (20), 10614-10619) or oligonucleotides synthesised directly onto glass using photo-lithographic techniques (Chee M. *et al*, (1996), Science 274 (5287), 610-61) to form what are commonly termed 'micro-arrays'.

In both methods the mRNA sequences extracted from control and test cells or tissues are either labelled directly or are first converted or amplified to yield equivalent cDNA sequences which are subsequently labelled. Once immobilised by hybridisation to complementary target sequences located on the array, the fluorescent labels attached to the probes are detected, either by scanning or by imaging, and quantified to yield data on the amounts of different mRNAs present in the test and control samples. Since sequences from test and control cells are labelled with different fluorophors, both samples can be applied and hybridised simultaneously and the resulting pattern and intensity of hybridised probes determined using detection instrumentation tuned to distinguish between the emission wavelengths of the fluorophors used.

Consequently these two-colour methods allow direct visualisation of differential expression of mRNAs between the two cell populations and are widely used in many fields of life science research to study the control and consequences of gene expression.

However, despite the relative simplicity and elegance of these methods, in operation they suffer from a number of technical difficulties which limit the ease of application and the speed of the techniques to the analysis of gene expression. The construction of target arrays is a time consuming and often expensive process requiring precision equipment: either for construction and alignment of the masks required for light directed oligonucleotide synthesis, or for precise application of nanolitre droplets of liquid for DNA arrays. Arrays based on DNA spots also suffer from artefacts arising from unequal application or drying of the very small volumes used, and often require

replicate spots to yield accurate data. With either type of array, if the user wishes to modify his investigations to include additional sequences a whole array must be constructed to accommodate the new targets. It would not be atypical to have to make an entirely new array simply to add a single new sequence to a pre-existing library of several thousand sequences.

The hybridisation process is also subject to a number of problems arising from the geometry of the system and the temperature required for the hybridisation process. To achieve sufficient sensitivity to detect low levels of mRNAs it is necessary to use a high concentration of labelled probe to achieve maximal hybridisation to target sequences; this requirement and the limited amount of probe material available results in hybridisation reactions being performed in very small volumes. Difficulties therefore arise in ensuring adequate coverage of arrays with microlitre quantities of solution, and the resulting thin films of liquid do not promote good access of the mobile probe sequences to the fixed target sequences; furthermore problems with evaporation are also common at the temperatures of 40°C to 65°C commonly used for hybridisation.

Finally, the detection and quantification of fluorophor-labelled probes on micro-arrays requires dedicated sophisticated equipment to detect the very low levels of fluorescence present. To achieve the required sensitivity, detection is most commonly achieved using a scanning laser spot to excite fluorophor molecules; this can be a very slow process requiring up to several hours to complete measurements from a single array.

This invention provides an alternative to micro-array systems for analysis of gene expression. Means are provided for performing analyses using a particle based technique so as to replace ordered 2D arrays with randomly oriented 3D arrays which can be quickly and easily modified to include new target sequences. The technique provides favourable geometry and kinetics for promoting efficient hybridisation, that can be performed in a standard reaction tube, and that allows measurement of hybridised probe to several thousand target sequences to be accomplished in a few seconds.

The invention provides a method of detecting and analysing differences between nucleic acids from two sources, which method comprises:

a. providing nucleic acids from two sources as labelled probes;

b. forming a mixture of the labelled probes with pooled reagents wherein

each reagent is a population of beads carrying a polynucleotide target, the target of one reagent being different from the target of another reagent, the beads of one reagent being distinguishable from the beads of another reagent;

c. incubating the mixture under conditions to promote specific hybridisation between probes and targets; and,

d. analysing beads in the mixture by flow cytometry.

A polynucleotide target is partly or wholly single-stranded and is capable of specific hybridisation. Oligonucleotides of at least 8 residues are preferred. Preferred are cDNA sequences derived, e.g. by RT-PCR amplification, from cellular mRNA.

The pooled reagents may comprise one bead, or preferably a plurality of beads, of each reagent.

Features of the invention include:

a) gene expression assays are performed on carrier beads;

b) individually identifiable beads or populations of beads each carrying a different target sequence are prepared;

c) selected beads, or populations of beads, are pooled together in suspension to provide a randomly oriented 3D array of particles carrying all sequences of interest for an individual investigation;

d) mRNAs or cDNAs prepared from control and test cells or tissues are labelled with fluorescent tags to identify their source;

e) labelled probe species are mixed with the pooled suspension of target carrying beads under conditions which promote specific hybridisation between probes and targets;

f) the bead mixture is analysed by flow cytometry to simultaneously determine the identity of each bead analysed (and hence the identity of the target sequence carried by the bead) and to quantify the amounts of both control and test probes bound to each bead; and

g) data is analysed to yield information on the relative and absolute abundance of each mRNA in the control and test samples.

The beads of one reagent can be distinguished from the beads of another reagent by a number of different means. Suitable distinguishing means include differences in size, colour or fluorescence or the nature or concentration of markers

attached to the beads. Beads of one reagent can be distinguished from beads of other reagents using one or more of such means.

For clarity, the invention is now described with reference to the following figures:

Figure 1: Schematic illustration of 2D ordered array and 3D random array.
Figure 2: Flowchart illustrating the principle of the bead based flow cytometry gene expression process.
Figure 3: Schematic representation of the bead based flow cytometry gene expression process.
Figure 4: Schematic representation of method for analysis of differential expression of TNF and GAPDH genes in LPS stimulated THP-1 cells.

With reference to Figure 1, in a two dimensional ordered array the locations of each target spot immobilised on a planar surface are defined by x,y co-ordinates and hence target sequences are identified by the same co-ordinates. In contrast, in a 3D array formed from particles dispersed in a space defined by dimensions x,y,z, if each particle is individually identifiable by some inherent characteristic, it is not necessary to use x,y,z locations to specify the identity of each bead and the particles can be randomly distributed throughout the volume as in a suspension of beads in liquid. It follows that if each bead is individually identifiable, then any target sequence previously coupled to that bead is also identifiable. Therefore if a number of differing beads, or discrete populations of beads, are individually prepared where each bead carries a different target sequence and then selectively pooled, the pooled beads can form a 3D array which can be used for gene expression analysis.

Beads suitable for use in the method of the invention are those which can be readily identified during analysis by flow cytometry; such beads have been previously developed and used for diagnostic assays to measure a wide range of analytes in blood and other biological fluids by immunoassay. A desire to have a higher throughput in these applications has led to the development of multiplex methods which allow more than one analyte to be measured simultaneously by means of flow cytometry analysis. Multiplexing is achieved by carrying out solid phase linked assays

using plastic or latex beads as assay substrates. By using a number of discrete bead types which are individually distinguishable from each other, where each bead type carries reagents for one assay, standard flow cytometer instrumentation may be used both to identify the bead type and to measure the assay signal associated with each bead. Discrimination between bead populations can be achieved by size (Frengen J. *et al* (1995), Journal of Immunological Methods, Volume 178, p141), by colour or fluorescence (Fulwyier M.J. UK Patent 1,561,042) or by electronic means (Mandecki W. US Patent 5,641,634).

The general principle of the process of the invention is now described with reference to Figure 2. Selected target cDNA sequences are prepared by standard PCR methods incorporating a means to allow coupling of target sequences to beads. One suitable method would utilise a 5'-biotin on one of the PCR primers, yielding a 5'-biotinylated DNA suitable for coupling to streptavidin-coated beads. Those skilled in the art will recognise that alternative chemical coupling strategies are available. Such alternative strategies may include, for example, synthesising oligonucleotides having a chemical group such as an amino group at the 5' end thus rendering them suitable for crosslinking to beads which have been modified to have, for example, carboxyl groups on their surface. It will also be appreciated that oligonucleotides synthesised with a terminal biotin or other coupling group could readily be used in place of PCR generated DNA sequences.

Once the required number of target cDNAs (cDNA 1 to cDNA n) have been prepared, each target sequence is separately coupled to a corresponding discrete population of beads (Bead 1 to Bead n respectively). Aliquots are then removed from each population and pooled to form a mixed suspension of beads constituting a randomly orientated 3D array of target sequences. The 3D array is then hybridised with fluorescently labelled probes (RNA or cDNA) prepared from the control and target cells or tissues which have been labelled with two different fluorophors (Fluor A and Fluor B respectively). Following hybridisation the mixed population of beads is analysed by flow cytometry; as each bead is analysed information from the flow cytometer detectors is used to identify the bead and to measure the amounts of Fluor A (control mRNA) and Fluor B (sample mRNA) bound to the complementary target sequence carried by the bead. These measurements are then used to determine the relative expression of each

mRNA in the samples.

In a further illustration of the method of the invention reference is made to one embodiment as shown in Figure 3. Control (1) and test (2) probes are prepared and labelled using standard methods and aliquots mixed in a tube (3) containing a mixture of beads (4) carrying the desired target sequences and the tube sealed. Hybridisation of probe and target sequences are promoted by incubating the mixture under conditions of heat, pH and salt concentration which are known to allow the formation of specific nucleic acid hybrids. Following hybridisation, the bead mixture is analysed by flow cytometry using multiple channel fluorescence detection. In the embodiment illustrated, two fluorescence channels are used to identify beads and two further channels are used to measure control and test probe fluorescence. For each bead passing through the flow cytometer this data produces a set of data values that can be represented as 3D plots for control probes (7) and test probes (6). Bead identity is determined by measuring the amounts of two different fluorophors (bead Fluor 1 and bead Fluor 2) incorporated within the bead during manufacture. Plotting the intensities of the two fluorophors on x,y axes (8 & 9) separates the different bead populations used.

The number of possible target sequences that can be measured in a single assay will necessarily be limited by the number of bead populations which it is possible to discriminate in a mixture. With current flow cytometry instrumentation this does not pose a limitation on the utility of the procedure. Typical modern flow cytometry instruments are capable of simultaneously measuring fluorescence at four wavelengths together with other parameters, for example light scattering which is a measure of the size of particles under analysis. In addition, the dynamic range of fluorescence detection is high and fluorescence may be accurately measured over several orders of magnitude. Given this sophistication in measurement it is relatively straightforward to devise schemes which yield a large number of individually distinguishable bead populations to serve as carriers. For example if beads are prepared which contain 2 separate fluorophors, with each fluorophor present in one of 10 levels, then $10^2 = 100$ bead types are created. By increasing the number of fluorophors, or the levels of each fluor or by introducing other variables, such as bead size, larger numbers of discrete bead types can be produced.

Plotting the intensity of probe fluorophors on the z axis of two different

plots (10 & 11) shows the amounts of the control (10) and test (11) probes bound to each bead population. This allows the production of a table of the amounts bound to each target sequence in the analysis (14). In the schematic example shown, one mRNA species (12) is expressed at a lower level in the test sample than in the control (13). Other differences in expression can be readily identified as differences in the heights of equivalent peaks in the two plots.

In micro-array technique applications DNA or oligonucleotide target sequences are typically applied to a solid surface as discrete areas of dimensions in the range 10-100 μm, with dimensions of 50-100 μm being typical of DNA spots applied as liquid droplets, and smaller areas being used in techniques utilising photo-lithographic oligonucleotide synthesis. To ensure accuracy in measurement of differential expression it is important that the amount of DNA or oligonucleotide present on the solid phase is in excess of complementary sequences in the probe solution such that target sequences do not become limiting leading to distortion of hybridisation results. Consequently it is crucial that in any procedure using flow cytometry for analysis of gene expression using target sequences carried on beads, that the capacity of the system retains the same degree of target:probe excess as used in conventional techniques. Beads used for flow cytometry typically have diameters in the range from 1-10 μm and therefore individually do not have sufficient surface area to substitute for a typical micro-array. However by using several beads to carry each target sequence it is possible to achieve equivalence in target presentation as shown in the following example:

For a 2D array with 1 00 μm ∅ spots:

spot area $= \pi r^2$

$= 3.14 \times (50)^2$

$= 7850\ \mu m^2$

For 10 μm ∅ beads in beam (50% surface illuminated):

lit area $= 2\pi r^2$

$= 2 \times 3.14 \times (5)^2$

$= 157\ \mu m^2$

Bead:Spot equivalence	= 7850 / 157
	= 50 beads

Bead volume (assuming cubic packing)	= 10^3 μm^3
Volume of 50 beads	= 50,000 μm^3
Assuming 10% v/v suspension	= 500,000 μm^3
	= $5 \times 10^5 / 1 \times 10^9$ μl
	= 1×10^{-3} μl
	= 1 nl

Consequently for a complete assay of 1000 targets using 50 beads/target the total volume required for the assay is 1 μl. If desired, larger volumes may be used for convenience in hybridisation or analysis; for example using beads at a concentration of 1 % v/v would give a 10 μl total volume.

If desirable, the method of the invention would allow larger numbers of beads to be used with a consequent increase in capacity for binding a greater mass of target sequence spread over the total bead population. This would allow the user if desired to increase the amount of probe bound to the bead populations to increase the sensitivity of the process for detecting rare species. Alternatively, it enables an increase in the number of samples which may be analysed simultaneously, for example, to measure expression of a panel of genes simultaneously in a control and more than one test sample, where as described previously each control or test sample is labelled with a different fluorophor. Such increases in assay complexity are not achievable with conventional arrays on solid surfaces without reducing sensitivity due to the finite capacity of array spots for binding complementary sequences.

It can be appreciated by the skilled worker that the method of the present invention provides a number of significant advantages over previously described procedures for gene expression analysis which are based on 2D arrays:

a) the basic components for the bead based assay are readily prepared by coupling solutions of cDNA or oligonucleotides to commercially available beads using standard coupling methods,

b) no specialised equipment is required for preparation or analysis, in contrast to the dedicated array production and scanning equipment required for micro-arrays,

c) the design of investigations can be easily modified with target sequences being added or deleted at will without the requirement to scrap existing materials,

d) hybridisation is performed in suspension in standard reaction vessels, thereby avoiding problems with evaporation associated with thin films of liquid covering micro-arrays and promoting hybridisation through efficient mixing of probe and target sequences, and

e) analysis speed is significantly improved: flow cytometers typically analyse beads at rates of 1,000-10,000 beads/second allowing processing of a 100 sequence gene expression analysis in a few seconds.

In a further illustration of the method of the invention reference is made to one embodiment as shown in Figure 4.

Figure 4 shows a scheme of a method for the analysis of differential Tumour Necrosis Factor (TNF) expression in THP-1 cells either treated in the presence (test) or absence (control) of bacterial lipopolysaccharide (LPS) (Su S. etal BioTechniques 1997, 22:1107-1113). In this method, variations in abundance of the TNF gene transcripts in control and test samples are simultaneously compared to levels of transcripts from the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in the same samples.

Briefly, human monocyte THP-1 cells at 5 x 10^5 cells/ml are treated in the presence (test sample) and absence (control sample) of 10μg/ml LPS for 90 minutes. Following treatment, both samples are processed separately to isolate the RNA populations present in the control and test samples according to standard procedures set out in Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory Press 1989, pp 7.3-7.87. Other suitable methods for RNA isolation will be recognised by someone skilled in the art and include the use of commercially available reagents or kits (e.g. RNeasy, Qiagen).

Following RNA isolation, mRNA molecules are converted to cDNA by means of the enzyme, reverse transcriptase, using the standard method set out in Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory Press 1989,

pp 8.11-8.13.

The cDNA molecules obtained from each sample are then used in separate multiplex PCR reactions using standard conditions (Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory Press 1989 pp 14.5-14.20) and primer pairs which amplify GAPDH and TNF (Su S. etal BioTechniques 1997, 22:1107-1113). One primer of each primer pair is modified at the 5′ end with a biotin molecule to aid in strand separation in subsequent steps in the process. This is readily achieved using standard techniques during oligonucleotide synthesis with modified phosphoramidites, for example : 5'-Biotin Phosphoramidite (Glen Research). Fluorescently labelled nucleotides are incorporated into the PCR reactions in order to label the amplified PCR products such that their origin from the test or control RNA populations may be established. Thus, the PCR reaction mix containing cDNAs prepared from the control cells includes Cy3™-dCTP and the reaction mix containing cDNAs prepared from test cells includes Cy5™-dCTP (Cy3™-dCTP and Cy5™-dCTP are obtained from Amersham Pharmacia Biotech). These reactions yield amplified levels of fluorescently labelled cDNA populations wherein the relative abundance of individual cDNA species corresponds to the abundance of their respective parent mRNA species in the RNA populations isolated from the cell or tissue samples and where the origin of any given molecule from the test or control sample populations can be ascribed from the fluorescent label carried by the molecule.

The PCR products from the control (Cy3-labelled) and test (Cy-5 labelled) reactions are now mixed together prior to further processing.

Double stranded cDNA products of the PCR reactions are converted to single stranded cDNA molecules prior to further analysis by binding the PCR products via the biotin at the 5' end of each PCR product to streptavidin-coated magnetic beads (MagneSphere, Promega). Once bound, the double stranded PCR products are denatured by addition of 0.2 volume of 2M NaOH, incubating for 10 minutes at room temperature in order to release the non-biotinylated strands from the beads. The beads are separated from the solution by attraction to a magnet and the clarified solution, which contains the single stranded labelled PCR products, is removed and made neutral by addition of 1 volume of 0.4M HCl. Other methods for separating double-stranded DNA molecules are known to those skilled in the art.

Populations of beads or particles suitable for performing analysis of

differential gene expression are prepared using beads containing fluorescent dyes (SPHERO™, Spherotech Inc.). Yellow fluorescent streptavidin-coated beads are coated with the 5′-biotin primer for GAPDH and blue fluorescent streptavidin-coated beads are coated with the 5'-biotin primer for TNF (using the same 5'-biotin primers as were used earlier). Thus, these populations of beads have two distinguishing characteristics i.e. each bead population is distinguishable from all other bead populations by its fluorescence characteristics and each bead population is capable of recognising a single cDNA species (either TNF or GAPDH).

The separately prepared beads are mixed together, further mixed with the Cy 3 and Cy 5-labelled single stranded cDNA products prepared previously and incubated in a hybridisation buffer (0.1M Tris.HCl pH7.4, 750mM NaCl) at 45° for 2 hours prior to analysis of the bead populations by flow cytometry. cDNA products from the mixed population will bind specifically to their complementary capture sequences carried on beads such that each bead will ultimately be decorated with a mixture of labelled cDNAs of a single species arising from the test and control populations, and where the relative abundance of the labels reflects the relative abundance of single species in the original test and control populations.

The relative abundance of GAPDH transcripts in the population of molecules derived from control and test samples is compared by detecting the fluorescence of yellow beads at 460nm/ 480nm (excitation/emission); and, within the population of yellow beads, bound Cy3 labelled transcripts are detected at 550nm/570nm (excitation/emission) and bound Cy5 labelled transcripts are detected at 650nm/670nm (excitation/emission). The relative abundance of TNF transcripts in the molecules derived from test and control samples is determined by detecting the fluorescence of blue beads at 650nm/710nm (excitation/emission); and further detecting bound Cy3 labelled transcripts at 550nm/570nm (excitation/emission) and bound Cy5 labelled transcripts at 650nm/670nm (excitation/emission). Thus bead fluorescence is used to assign the identity of the gene associated with each bead and label fluorescence is used to determine the relative abundance of cDNAs arising from the test and control samples which are attached to the bead, the latter yielding information on the differential expression of that gene under the conditions used in treatment of the original cell or tissue sample.

CLAIMS

1. A method of detecting and analysing differences between nucleic acids from two sources, which method comprises:

a. providing nucleic acids from two sources as labelled probes;

b. forming a mixture of the labelled probes with pooled reagents wherein each reagent is a population of beads carrying a polynucleotide target, the target of one reagent being different from the target of another reagent, the beads of one reagent being distinguishable from the beads of another reagent;

c. incubating the mixture under conditions to promote specific hybridisation between probes and targets; and,

d. analysing beads in the mixture by flow cytometry.

2. The method of claim 1 wherein the nucleic acids from two sources are mRNA or cDNA from cells or tissues.

3. The method of claim 1 or claim 2 wherein the polynucleotide targets are cDNA derived from cellular mRNA.

4. The method of any one of claims 1 to 3 wherein the polynucleotide targets are PCR amplimers.

5. The method of any one of claims 1 to 4 wherein the polynucleotide targets carry terminal biotin groups through which they are attached to streptavidin-coated beads.

6. The method of any one of claims 1 to 5 wherein the polynucleotide targets are single-stranded nucleic acids.

7. The method of any one of claims 1 to 6 wherein the labelled probes are single-stranded nucleic acids.

8. The method of any one of claims 1 to 7 wherein beads of one reagent are distinguishable from beads of another reagent by size.

9. The method of any one of claims 1 to 8 wherein beads of one reagent are distinguishable from beads of another reagent by the nature of the markers attached to the beads.

10. The method of any one of claims 1 to 9 wherein beads of one reagent are distinguishable from beads of another reagent by the concentration of markers attached to the beads.

11. The method of any one of claims 1 to 7 wherein beads of one reagent are distinguishable from beads of another reagent by size and/or by the nature and/or the concentration of markers attached to the beads.

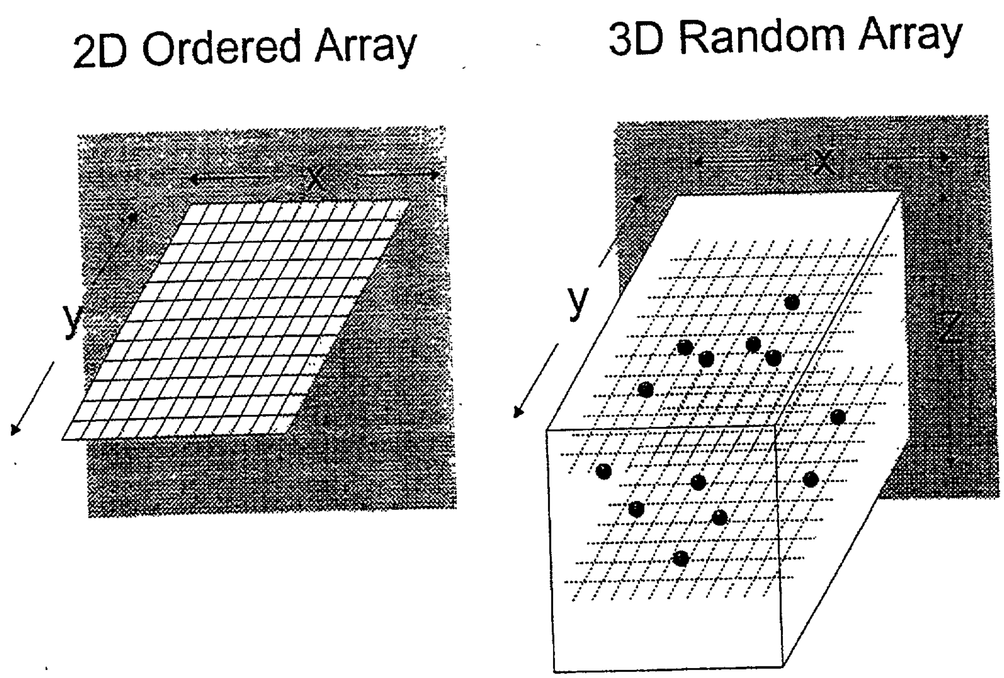
12. The method of any one of claims 8 to 11 wherein fluorescent markers are attached to the beads.

13. The method of claim 1 or claim 2 wherein each probe is labelled with a fluorescent tag to indicate its source.

14. The method of any one of claims 1 to 13 wherein analysis by flow cytometry is performed to identify each bead and to quantify the probes bound thereto.

15. The method of any one of claims 1 to 14 wherein data obtained by flow cytometry is analysed to yield information about the relative and/or absolute abundances of individual sequences of the nucleic acids from the two sources.

Figure 1.



09/914603

Figure 2

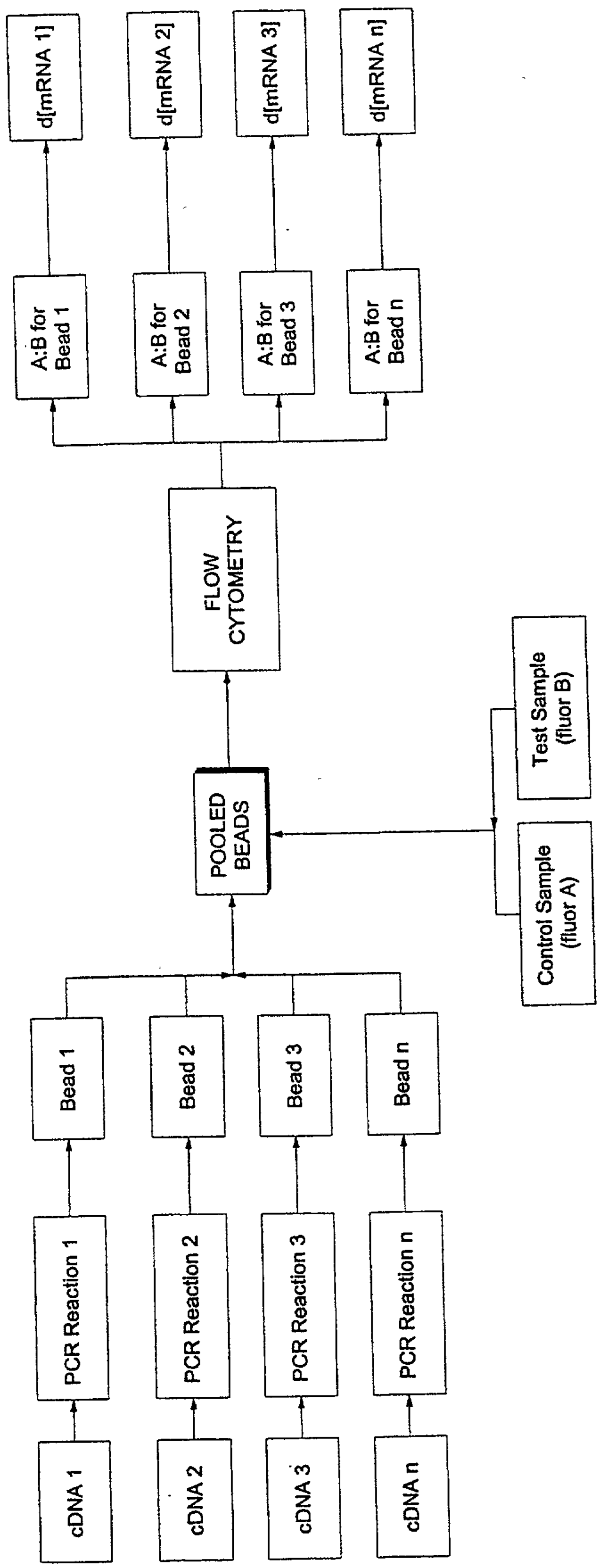


Figure 3.

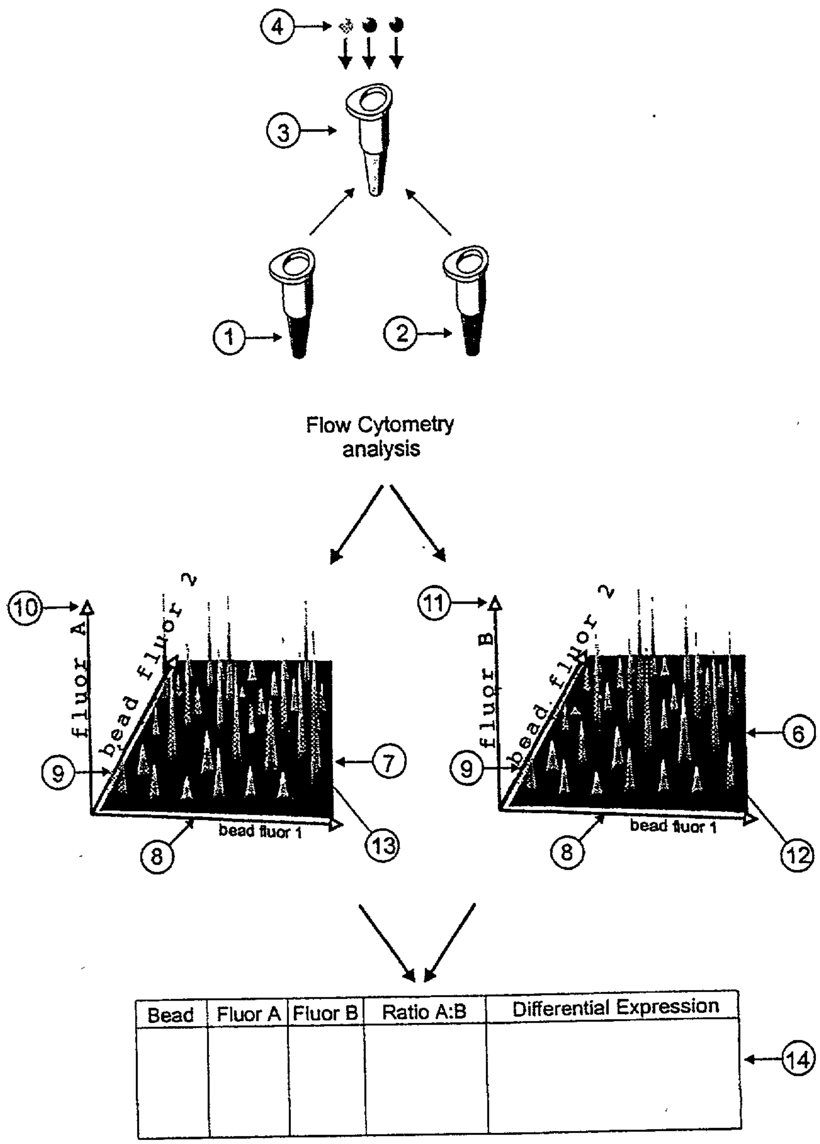
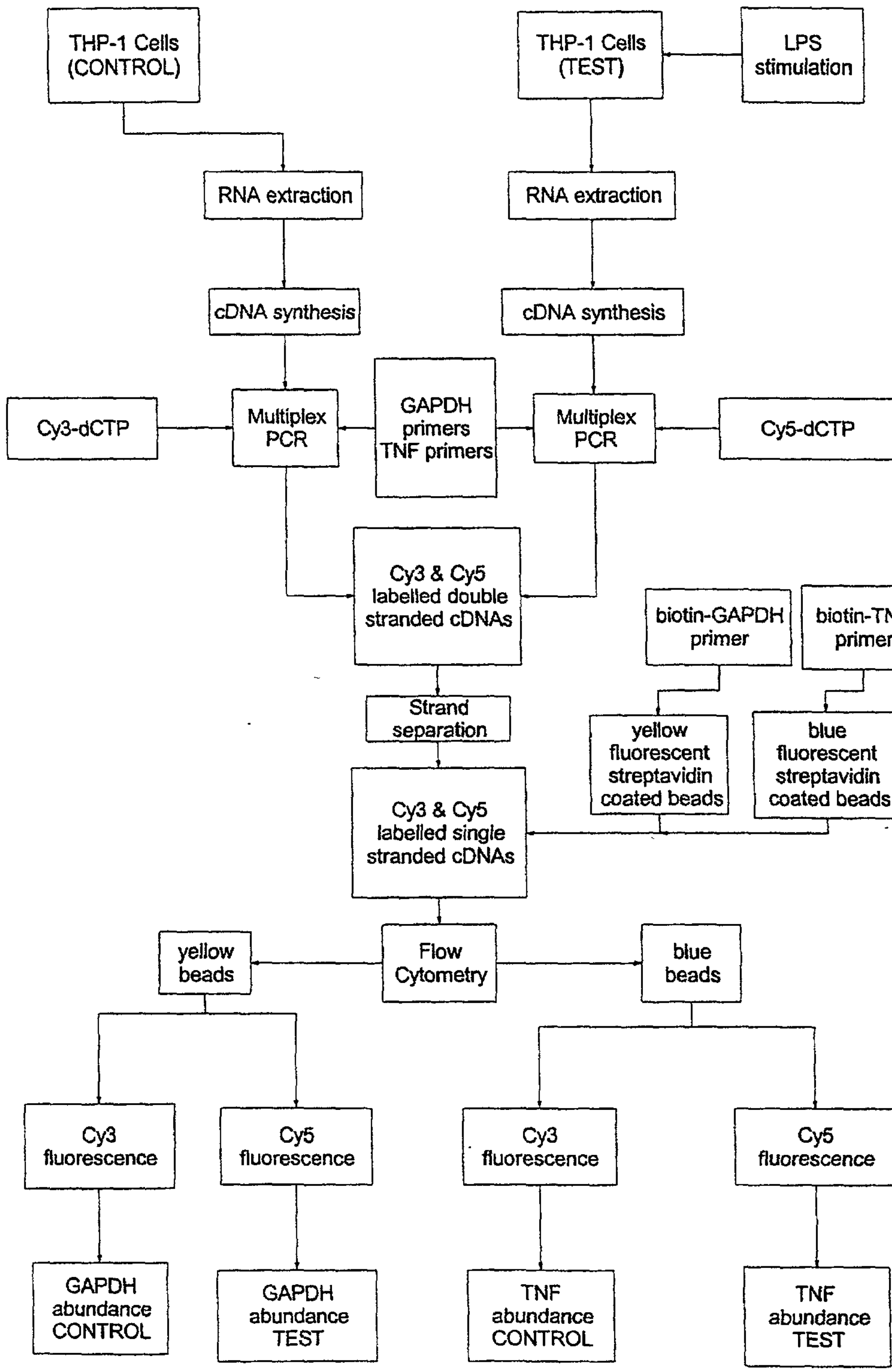

4
3
1
2
Flow Cytometry analysis
10
11
fluor A
fluor B
bead fluor 2
bead fluor 1
9
7
6
8
13
12
Bead
Fluor A
Fluor B
Ratio A:B
Differential Expression
14

Figure 4. Analysis of differential expression of TNF & GAPDH genes in LPS stimulated THP-1 cells



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PTO/SB/01 (12-97)
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Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted with Initial Filing OR ☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	PA-9902
First Named Inventor	Thomas
COMPLETE IF KNOWN	
Application Number	09 /914,603
Filing Date	28-Aug-2001
Group Art Unit	To be assigned
Examiner Name	To be assigned

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Analysis of Differential Gene Expression

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) 08/28/2001 as United States Application Number or PCT International Application Number 09/914,603 and was amended on (MM/DD/YYYY) ________ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
9905807.5	Great Britain	03/12/1999	☐	☐	☒
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C 119(e) of any United States provisional application(s) listed below

Application Number(s)	Filing Date (MM/DD/YYYY)	
		☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

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Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION — Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*
PCT/GB00/00807	03/09/2000	

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: ☒ Customer Number 22840 → Place Customer Number Bar Code Label here 22840
OR
☐ Registered practitioner(s) name/registration number listed below

PATENT TRADEMARK OFFICE

Name	Registration Number	Name	Registration Number

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☒ Customer Number or Bar Code Label 22840 *OR* ☐ Correspondence address below

Name					
Address					
Address					
City		State		ZIP	
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Nicholas	Thomas

Inventor's Signature						Date	
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City		State		ZIP		Country	

☒ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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As a below named inventor, I hereby declare that:

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I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the *invention entitled:*

Analysis of Differential Gene Expression

(Title of the Invention)

the specification of which

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9905807.5	Great Britain	03/12/1999	☐	☐	☒
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

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I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below

Application Number(s)	Filing Date (MM/DD/YYYY)	
		☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

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U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*
PCT/GB00/00807	03/09/2000	

☐ Additional U.S or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: ☒ Customer Number 22840 → Place Customer Number Bar Code Label here

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Country		Telephone		Fax	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])				Family Name or Surname	
Nicholas				Thomas	
Inventor's Signature				Date	30/10/01
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City	GB3	State	ZIP	Country	

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☒ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 1 of 1

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

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Alan	Waggoner

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Given Name (first and middle [if any])	Family Name or Surname

Inventor's Signature						Date	
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DECLARATION	ADDITIONAL INVENTOR(S) Supplemental Sheet Page 1 of 1

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Post Office Address							
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Post Office Address							
Post Office Address							
City		State		ZIP		Country	